

DIFFERENTIAL REGULATION OF THE UNCOUPLING PROTEIN 1
GENE

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DIFFERENTIAL REGULATION OF THE UNCOUPLING PROTEIN 1
GENE

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Abstract

The mouse uncoupling protein 1 gene (*Ucp1*) is expressed specifically in brown adipose tissue and its transcription is increased by cold exposure. We have analyzed the transcriptional control of this gene through deletion analysis of the upstream untranslated regions of *Ucp1*. Mutated minigene constructs were microinjected into mouse pronuclei to generate transgenic mice. Positive transgenic founders were crossed with Balb/cByJ +/+ mice to generate F₁ offspring heterozygous for the transgene, which were then examined for tissue-specific and cold inducible expression of the minigenes by northern analysis. Protein-DNA interactions at putative regulatory sites were also explored through *in vitro* gel shift assays of the promoter and enhancer regions. We found that the sequence between -2,581 and -2,335 bp of the enhancer region is critical for the expression of *Ucp1*. We also discovered that the region located between -991 and -230 bp does not play a major role in *Ucp1* expression. We found that nuclear factor-1 does not play a regulatory role at -166 bp in the promoter region. It was also observed that CCAAT enhancer binding protein does not interact with the 5' flanking region at -2,450 to -2,411 bp.

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1. Introduction

Uncoupling protein 1 (UCP1) is a 32 kDa mitochondrial inner membrane protein that has six transmembrane domains (Nicholls and Locke, 1984). UCP1 functions as a fatty acid coupled proton transporter (Garlid et al., 1998). UCP1 dissipates the proton gradient by uncoupling substrate oxidation from ATP synthesis. Dissipation of the proton gradient produces heat by a process known as non-shivering thermogenesis. The activity of UCP1 is inhibited by purine nucleotides binding on the cytosolic side of the mitochondrial inner membrane, resulting in inhibition of its transport function (Gonzalez-Barroso et al., 1998). Fatty acids increase UCP1 activity either by serving as a fatty acid anion transporter or by acting as a prosthetic group of UCP1 (Gonzalaez-Barroso et al., 1998). UCP1 mediated non-shivering thermogenesis is unique to brown adipose tissue (BAT). Although UCP1 is only found in BAT, several other uncoupling proteins have been identified in various tissues of both rodents and humans. UCP2 is present in a large number of human tissues, with highest levels in skeletal muscle and white adipose tissue (WAT) (Fleury et al., 1997). UCP3 is abundant in skeletal muscle and BAT and is regulated by diet, thyroid hormone, β 3-adrenergic agonists and leptin, suggesting a role in regulation of energy balance (Gong et al., 1997). UCP4 is a brain specific UCP homolog (Mao et al., 1999). Another uncoupling protein homolog known as brain mitochondrial carrier protein-1 (BMCP1), has been found in the central nervous system and at much lower levels in peripheral tissues (Sanchis et al., 1998). All of these proteins are structurally related to UCP1, but are expressed in diverse tissues and may be uniquely

regulated (Denjean et al., 1999; Carmona et al., 1998). Due to the recent discovery of these proteins, we know very little about their gene-regulatory mechanisms. Therefore, elucidation of the uncoupling protein 1 (*Ucp1*) gene regulation in BAT should provide meaningful baseline data for comparison to the remaining *Ucp* homologs.

BAT thermogenesis has two functions: to maintain body temperature in response to cold and to maintain energy balance following excess caloric intake (Himms-Hagen et al., 1994). Genetic deletion of BAT in mice results in obesity from an increase in metabolic efficiency (Lowell et al., 1993). In addition, expression of the *Ucp1* gene in white adipose tissue (WAT) results in a decrease in white adipose tissue mass and resistance to obesity (Kopecky et al., 1996). It has also been shown that *Ucp1* expression can be increased in response to dietary fat challenge and suggests that a high fat diet selectively regulates *Ucp1* expression in BAT (Surwit et al., 1998). Collectively, these studies indicate that *Ucp1* gene expression is critical in terms of energy balance and therefore obesity.

1.1 *Ucp1* Gene Regulation

Non-shivering thermogenesis is primarily regulated through increased transcription of *Ucp1* and activation of the UCP1 protein in response to cold stress. Norepinephrine binds to β_1 and β_3 adrenergic receptors on the brown fat cell surface and results in an increase in intracellular cyclic AMP (cAMP) which leads to an increase in the transcription of *Ucp1*. Three mechanisms are responsible for the rapid increase in levels of *Ucp1* mRNA in cold exposed rodents: an increase in the rate of initiation of *Ucp1*

transcription, an increase in the fraction of *Ucp1* transcripts that undergo elongation, and stabilization of the mature *Ucp1* mRNA (Rehnmarm et al., 1992).

In this study, we investigated the regulation of *Ucp1* mRNA transcription. Other studies have shown that regulatory elements needed for tissue-specific and cold induced *Ucp1* expression are located within -2.8 kb of the mouse *Ucp1* gene (Boyer and Kozak, 1991).

To define the regulatory regions that influence *Ucp1* expression, Boyer and Kozak generated a transgenic minigene (missing exons three, four and five) with 3 kb of 5' flanking DNA and 300 bp of 3' flanking DNA, known as *Ucp1*^{tg1}. Their work determined that the regulatory elements required for tissue-specificity and cold inducibility of the endogenous *Ucp1* gene were present in the *Ucp1*^{tg1} minigene. From the *Ucp1*^{tg1} construct, they deleted 1.8 kb from the 5' end of *Ucp1*^{tg1} creating *Ucp1*^{tg2} (Figure 1). This deletion resulted in the loss of expression of the *Ucp1*^{tg2} minigene in BAT, suggesting that there are essential regulatory elements between -3,000 and -1,200 bp of the 5' flanking DNA of *Ucp1* (Boyer and Kozak, 1991). Additional studies using transgenic rats have shown that regulatory elements needed for tissue-specific and cold induced *Ucp1* expression are located within -4.5 kb of the 5' flanking region of the rat *Ucp1* gene (Cassard-Doulcier et al., 1993). In the same study, chloramphenicol acetyl transferase (CAT) reporter assays were used to test the activity of the *Ucp1* promoter and enhancer regions and revealed several cAMP response elements within the -4.5 kb flanking DNA of the rat *Ucp1* gene. Cyclic AMP response elements (CRE) of the rat *Ucp1* gene are located at -4,218, -3,639, -2,512, -2,122, -1,850, -1,064 and -158 bp.

Boyer and Kozak also identified four CRE's in the 5' flanking DNA of the mouse *Ucp1* gene. Cassard-Doulcier et al. (1994) also revealed regulatory elements including an enhancer region at -2.3 kb, a minimal promoter at -157 bp, and a potential suppressor between -400 and -157 bp. DNase 1 footprinting of the enhancer region of the rat *Ucp1* gene revealed two domains at -2,444 to -2,423 bp and -2,352 to -2,319 bp. The first domain was shown to bind the retinoid X and triiodothyronine (T₃) receptors and the second was shown to cooperatively bind nuclear factor-1 (NF-1) and Ets1 (Cassard-Doulcier et al., 1994). Five additional footprints were reported in the 5' flanking region and include: region A, -509 to -472 bp corresponding to a repeated CACCC box; region B, -403 to -350 bp corresponding to a protein related to Est1; region C, -182 to -159 bp corresponding to a protein related to NF-1; region D, -147 to -120 bp which is able to bind *in vitro* proteins related to a cAMP response element-binding protein; and region E, -111 to -85 bp which binds Sp1 *in vitro* (Cassard-Doulcier et al., 1994). Another study investigating the direct effects of T₃ stimulation of the *Ucp1* gene in rats demonstrated that the thyroid hormone response sequence between -2,317 and -2,399 bp contains thyroid hormone response elements (TRE), which directly stimulate *Ucp1* (Rabelo et al., 1995).

Kozak et al. (1994) identified a 220 bp enhancer region in the 5' flanking region of the mouse *Ucp1* gene. In this region, they identified brown fat regulatory elements (BRE), which are important for tissue-specific expression and necessary for cAMP responsiveness. In addition, they defined two cAMP response elements; one near the

promoter and one further upstream. The upstream CRE3 element accounted for the responsiveness to cAMP and BRE1 was essential for the function of the CRE (Kozak et al., 1994).

In vivo footprinting of the 5' flanking region of *Ucp1* identified several potential regulatory regions and nuclear factors that may control the tissue-specific and cold inducible expression of *Ucp1* (Chen, 1996). The promoter region, which extends from the transcriptional start site to -272 bp, contains a CRE4 footprint at both -134 and -136 bp and a potential nuclear factor-1 response element at -166 bp. A 220 bp enhancer region located between -2,380 and -2,600 bp contains a footprint at a CRE3 site located at -2,563 and -2,555 bp, a peroxisome proliferator-activated receptor γ (PPAR γ) footprint located at -2,534 and -2,539 bp, a previously unidentified CCAAT enhancer binding protein (C/EBP) footprint located at -2,432 bp and a TRE footprint located at -2,397 bp (Chen, 1996). In summary, the *in vivo* DNase 1 footprinting experiments performed by Chen (1996) identified several footprints that confirmed previously observed nuclear factors and elucidated two novel footprints that were not previously identified, namely NF-1 and C/EBP (Figure 2).

1.2 Current Research

In order to further define the control of *Ucp1* transcription, the current study characterized the physiological significance of several protein-DNA binding motifs, the 5' flanking DNA of *Ucp1*, and the previously identified DNA binding motifs in the 5' flanking region of *Ucp1*. We utilized both *in vitro* and *in vivo* techniques to elucidate the

regulatory regions and nuclear factors that are critical for expression of this gene.

Functional analysis of *in vivo* footprinted protein-DNA interaction sites was performed by creating several transgenic mouse lines (Figure 1). The *Ucp1*^{tg3.1 and 3.2} line had a mutation in the potential NF-1 binding site located in the promoter region. The *Ucp1*^{tg4} line contained a deletion in the proposed enhancer region, -2,581 to -2,335 bp. The *Ucp1*^{tg6} line contained a deletion of the proposed silencer region, -272 to -900 bp. These transgenic mice were designed in order to answer three questions: (i) Will deletions within protein-DNA binding motifs or regulatory regions eliminate transcription of *Ucp1* in BAT of cold exposed (CE) mice? (ii) Do these deletions allow *Ucp1* to be expressed in mice adapted to thermoneutrality? (iii) Do these deletions result in expression of *Ucp1* in tissues other than BAT?

We used gel shift analyses of the promoter and enhancer regions of *Ucp1* to determine if the *in vivo* footprints of these regions correspond to actively bound tissue-specific nuclear factors. Oligonucleotides were synthesized to correspond to the footprints previously found through *in vivo* footprinting of the 5' untranslated flanking sequence of the *Ucp1* gene (Chen, 1996). Nuclear extracts were prepared from several different tissues in order to elucidate the tissue-specificity of potential DNA binding motifs in the promoter and enhancer region that were represented by the synthesized oligonucleotides (Table 1). Gel shift assays were performed on these oligonucleotides to determine the affinity of cold exposed brain, cold exposed liver, cold exposed BAT, and thermoneutral (TN) BAT nuclear extracts to the following *in vivo* footprinted sites: CRE3, PPAR γ , CRE2, C/EBP,

TRE and BRE2 in the enhancer region, and NF-1 and CRE4 in the promoter region. In order to further define two of the footprinted regions, corresponding to nuclear factor-1 (NF-1) and CCAAT enhancer binding protein (C/EBP), we performed additional gel shift assays utilizing specific antibodies and oligonucleotides. These experiments were aimed at characterization of the sequence elements that the nuclear factors were binding in the gel shift assay.

2. Materials and Methods

2.1 Animal Treatment

Balb/cByJ and transgenic mice were housed at room temperature and given Purina Rodent Chow and water ad libitum. To minimize transcription of the endogenous *Ucp1* gene and the transgene, mice were housed at 29°C for three days. These mice are referred to as thermoneutral (TN) adapted mice. To maximize *Ucp1* transcription, TN adapted mice were exposed individually to 4°C for one hour in pre-cooled cages with minimal bedding (to prevent huddling and maximize cold exposure). This has been shown to result in maximal transcription of *Ucp1* (Rehmark et al., 1992). These mice are referred to as cold exposed (CE). Mice were sacrificed by cervical dislocation and tissue samples were either frozen in liquid nitrogen or processed immediately. For investigations of tissue-specific mRNA detection by northern analysis, transgenic mice were exposed to 4°C for 24 hours in separate cages with minimal bedding to maximize *Ucp1* mRNA levels.

2.2 RNA Isolation and Northern Blot Analysis

Total RNA was prepared using the guanidinium isothiocyanate extraction method (Chomczynski and Sacchi, 1987) or with Tri reagent (Sigma). RNA was separated by formaldehyde fractionation gel electrophoresis and blotted onto Hybond N+ (Amersham) membrane as described by the manufacturer. The probes for northern blotting were prepared as a PstI/KpnI restriction digest of plasmid DNA containing a 500 bp fragment of the *Ucp1* gene and was labeled with [³²P] dCTP by random prime labeling (Boehringer

Mannheim). To monitor total RNA loading consistency, the northern blots were reprobed with a BamHI/EcoRI restriction fragment derived from the mouse 18S rRNA gene (Bowman et al., 1981).

2.3 Nuclear Extract Isolation and Gel Mobility Shift Assay

Nuclear extract was isolated from interscapular BAT of CE and TN mice and from the liver and brain of CE mice. Tissues were removed and flash frozen in liquid nitrogen then ground utilizing a mortar and pestle and transferred to a homogenizer with a homogenization buffer: 10 mM HEPES, pH 7.6; 15 mM KCl; 0.15 mM Spermine; 0.5 mM Spermidine; 1 mM EDTA; 20 mM Na_2HPO_4 , pH 7.2; 5 mM NaF; 0.2 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$; 0.5 mM DTT; and 0.2 mM PMSF. The homogenate was centrifuged at 500 X g at 0°C for five minutes. The resulting nuclei were lysed for 20 minutes on ice in a buffer containing: 20 mM HEPES, pH 7.6; 25% glycerol; 420 mM NaCl; 1.5 mM MgCl_2 ; 0.2 mM EDTA; 20 mM Na_2HPO_4 , pH 7.2; 5 mM NaF; 0.2 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$; 0.5 mM DTT; and 0.2 mM PMSF. Samples were stored in liquid nitrogen. Gel shift analysis was performed as described by Boyer and Kozak (1991). Synthetic oligonucleotides were synthesized with 5' overhangs by DNagency (Table 1) and were labeled with [^{32}P] dCTP by end filling with Klenow polymerase (New England Biolabs).

3. Results

3.1 Cold Exposed and Tissue-specific Northern Blot Analysis of Transgenic Lines

A map of the transgenic constructs we developed can be seen in Figure 1. The first and second transgenes were created by Boyer and Kozak (1991) to identify tissue-specific and cold inducible regulatory regions. *Ucp1*^{tg1} contains 3 kb of the 5' flanking untranslated DNA and 300 bp of the 3' flanking DNA. In order to distinguish between the endogenous mouse *Ucp1* gene and the transgene for northern and Southern blot analysis, exons three, four and five were removed to make the *Ucp1*^{tg1} minigene (Boyer and Kozak, 1991). The regulatory elements required for tissue-specific and cold inducible expression of the endogenous *Ucp1* gene are present in the *Ucp1*^{tg1} transgene.

Subsequent deletions described in this thesis were generated from the *Ucp1*^{tg1} construct and therefore, the original *Ucp1*^{tg1} transgene serves as our positive control. As shown in Figure 3, the nontransgenic Balb/cByJ mice have only one band corresponding to the endogenous *Ucp1* mRNA present in the CE BAT. In contrast, the positive transgenic shows two bands: the higher molecular weight band corresponds to the *Ucp1* mRNA and the lower molecular weight band corresponds to the *Ucp1*^{tg1} transgene mRNA. Tissue-specific analysis of the *Ucp1*^{tg1} transgene mRNA reveals that the transgene is only expressed in BAT, similar to the expression pattern of the endogenous gene (Figure 4). We compared expression of the remaining transgenic constructs to the expression of the *Ucp1*^{tg1} transgene (Figures 3 and 4). From the *Ucp1*^{tg1} construct, a deletion of 1.8 kb from the 5' end of *Ucp1*^{tg1} created *Ucp1*^{tg2} (Figure 1). This deletion resulted in total loss

of expression of the *Ucp1*^{tg2} minigene in BAT (Boyer and Kozak, 1991).

Ucp1^{tg3.1} and *Ucp1*^{tg3.2} were created by introducing a mutation into the *Ucp1* promoter region corresponding to the previously identified NF-1 DNA binding motif, using the Clontech site-directed mutagenesis kit. Although these are the same mutations, these lines are derived from different founder mice. This mutation, located at -166 to -163 bp, changed the endogenous *Ucp1* sequence from GGC to TTA. Previous research has shown that this simple three base pair mutagenesis results in the complete loss of NF-1 binding (Mermod et al., 1989). Mutation in this region of the promoter of the *Ucp1*^{tg3.1} and ^{3.2} transgenes resulted in no changes in expression of either *Ucp1*^{tg3.1} or *Ucp1*^{tg3.2}; there is still expression of the minigenes in the CE BAT (Figure 5 and 6). Analysis of the tissue-specific expression of the *Ucp1*^{tg3.2} minigene revealed that the transgene is expressed only in BAT (Figure 7).

Ucp1^{tg4} was made by removing 246 bp from the enhancer region of *Ucp1*^{tg1} (Figure 1). Using the Clontech site-directed mutagenesis kit, Mlu I restriction enzyme sites were generated at -2,581 and -2,335 bp from the transcription start site, then the 244 bp fragment was removed with a Mlu I digestion and the remaining DNA was self-ligated. We observed a complete loss of transgene expression and only the endogenous gene can be observed in CE BAT (Figure 8). These results demonstrate that the 246 bp deletion in the enhancer region completely eliminates the expression of the *Ucp1*^{tg4} construct. This finding was supported by the tissue-specificity northern blot, which shows that only the endogenous *Ucp1* is expressed in CE BAT while *Ucp1*^{tg4} is not expressed (Figure 9).

Analysis of *Ucp1*^{tg6}, the minigene with the proposed silencer deletion is shown in Figures 10 and 11. This transgenic line was created using the Clontech site-directed mutagenesis kit, through the introduction of Mlu I sites at -991 and -230 bp from the transcription start site. This 760 bp fragment was removed via Mlu I digestion and the DNA was self-ligated. Figure 10 shows that *Ucp1*^{tg6} transgenic animals did not express the transgene differently than the endogenous *Ucp1* (Figure 3). The tissue-specificity blot shows *Ucp1*^{tg6} was expressed exclusively in BAT (Figure 11).

3.2 Analysis of the Gel Shifts from the Enhancer Region

In vitro gel shift assays were conducted in order to characterize nuclear factors that bound the *in vivo* footprinted regions found by Chen (1996). All gel shifts were carried out with the same preparations of nuclear extracts isolated from BAT, liver and brain. Ten µg of corresponding nuclear extract were added per lane. All competition reactions contained 200-fold molar excess unlabeled oligonucleotide. All probes and competitors used in the gel shifts are shown in Table 1.

Figure 12 shows a gel shift assay using an oligonucleotide derived from a section of the enhancer that contains the hypersensitivity site that corresponds to CRE3 (-2,586 to -2,547 bp; GSAP1). All nuclear extracts produced a single shift, 200-fold molar excess unlabeled GSAE1 resulted in a partial competition with the labeled probe. The strongest shift was produced with CE liver extract followed by TN BAT and CE brain extract and the weakest shift was produced by CE BAT. A shift indicates that a nuclear factor has bound the oligonucleotide and has retarded its migration through the gel matrix during

electrophoresis.

Figure 13 represents a gel shift assay using GSAE2, a section of the enhancer region between -2,552 and -2,513 bp that contains the hypersensitivity site to PPAR γ and CRE2. All of the extracts produced shifts. The strongest was produced by CE liver extract followed by extract obtained from TN BAT. CE BAT extract was the next strongest shift followed by CE brain extract, which produced two shifts. All shifts were partially removed with the addition of 200-fold molar excess unlabeled GSAE2.

Figure 14 is a gel shift assay of the GSAE3 oligonucleotide probe containing -2,450 to -2,411 bp of *Ucp1*, a region of the enhancer corresponding to the binding site of C/EBP. We observed that TN BAT extract resulted in the strongest shift followed by CE liver extract and then CE BAT extract. CE brain extract showed a very weak shift. All competitions, with 200-fold molar excess unlabeled GSAE3, resulted in partial or complete removal of the bands.

The competition assay using GSAE4, a region of the enhancer that correlates to the hypersensitivity sites for TRE and BRE2 (-2,416 to -2,377 bp), is shown in

Figure 15. The strongest shift was seen in CE brain, followed by CE liver then TN BAT. The weakest shift occurred with CE BAT. Two hundred-fold molar excess unlabeled GSAE4 resulted in complete removal of the shifts.

Figure 16 is a competition assay that corresponds to the suspected C/EBP hypersensitivity site of the enhancer found by Chen (1996). The negative control contains labeled GSAE3 without nuclear extract. The positive control contains labeled GSAE3 with CE BAT

extract and resulted in two shifts. All the remaining lanes contain 10 μ g of CE BAT extract and competitors or antibodies. The third lane shows the effect of the addition of 200-fold molar excess unlabeled GSAE3, which resulted in the partial removal of the higher molecular weight band while the lower molecular weight band is almost completely removed. To further characterize the proteins that are binding GSAE3, we competed the shift in the third lane with a mutated C/EBP binding motif in the GSAE3 oligonucleotide (mC/EBP). Although a return of the shift was expected, we observed only a partial competition of the lower band. The fifth lane shows that when competed with a consensus C/EBP oligonucleotide, no competition was observed. With the addition of C/EBP antibodies α , β and δ in the sixth, seventh and eighth lanes, respectively, no supershifts were observed, suggesting that C/EBP proteins were not binding to the GSAE3 oligonucleotide. The ninth and tenth lanes correspond to GSAE3 that has been mutated on either the right or left flanking regions of the suspected hypersensitivity site of C/EBP (Table 1). We observed that no competition occurred in GSAE3mR, but the lower band is removed with the 200-fold addition of GSAE3mL, suggesting that proteins interacted with the right half of the oligonucleotide flanking the C/EBP hypersensitivity site.

3.3 Analysis of the Gel Shifts from the Promoter Region

Figure 17 is a competition assay of the probe GSAP1, a section of the promoter region that corresponds to the NF-1 DNA binding site. Nuclear extracts from several tissues bound to the GSAP1 oligonucleotide. The strongest shift was observed with CE liver

extract, followed by the CE BAT extract, then the TN BAT extract and lastly the CE brain extract. Addition of 200-fold molar excess competitor DNA (unlabeled GSAP2) resulted in minimal removal of the shifts.

A gel shift assay using the probe GSAP2 is shown in Figure 18. This region of the promoter corresponds to the CRE4 binding site and resulted in strong shifts from all nuclear extracts. Two-hundred fold molar excess unlabeled GSAP2 partially removed the shifts.

Figure 19 is a competition assay of GSAP1, which corresponds to the NF-1 DNA binding motif in the promoter region found by Chen (1996). The negative control, the first lane, consists of labeled GSAP1 without nuclear extract. The second lane, the positive control, consists of labeled GSAP1 with 10 μ g CE BAT extract. The remaining lanes contain 10 μ g CE BAT extract and 200-fold molar excess unlabeled competitors or NF-1 antibody. The third lane contains unlabeled GSAP1, which competes the shift away, however, DNA with a mutation of the hypersensitivity site in the NF-1 binding motif (mGSAP1) also competed the shift. Competition with NF-1 antibody resulted in no supershift.

GSAP1mR and GSAP1mL are sequences that have mutated flanking regions around the NF-1 hypersensitivity site. Only the GSAP1mR competitor resulted in a decrease in the intensity of the shift.

4. Discussion

4.1 C/EBP and NF-1 Effects on *Ucp1* Expression

NF-1 has been implicated in regulation of adipocyte specific genes (Graves et al., 1991).

Although *in vivo* footprinting suggests that nuclear factors are binding the *Ucp1* promoter (Chen 1996), gel shift analysis failed to confirm that NF-1 or other specific protein-DNA interactions were binding to the region between -166 and -163 bp. In support of the gel shift assays in this region (Figure 12), analysis of the transgenic line *Ucp1*^{tg3}, containing a deletion of the NF-1 DNA binding motif, between -166 and -163 bp, results in normal tissue-specific and cold inducible expression of the *Ucp1* gene and suggests that this NF-1 binding motif is not involved in the regulation of *Ucp1 in vivo*. It is possible that a protein containing a binding motif similar to NF-1 is interacting with our oligonucleotide and binding to the flanking region extending to the right of the NF-1 binding motif resulting in the hypersensitivity site and *in vitro* gel shifting. It is also possible that the binding is non-specific, because of the inability of competitors to remove the shift.

The C/EBP family of transcription factors has been associated with both the differentiation of BAT from preadipocytes (Darlington et al., 1998) and the expression of *Ucp1* (Manchado et al., 1994). The C/EBP family has many isoforms that are present in several tissue types. Moreover, it has been shown that C/EBP β , but not C/EBP α , is increased in rat BAT after one hour of cold exposure, at both transcriptional and translational levels (Yubero et al., 1994), suggesting that the C/EBP family of proteins is tightly coupled with *Ucp1* regulation.

Following the identification of the *in vivo* footprint occurring at a C/EBP binding motif located between -2,450 and -2,411 bp, we performed a gel shift competition assay with a labeled oligonucleotide from this region, GSAE3 (Figure 16). Although C/EBP has previously been implicated in the control of *Ucp1* gene expression, our results do not strongly support the idea that this region of the enhancer contains a functional C/EBP binding site (Figure 16).

4.2 Nuclear Factors Bind to Additional Footprinted Regions *In vitro*

To determine whether nuclear factors bound to the additional sites previously identified through *in vivo* footprinting (CRE's, PPAR γ and TRE) (Figure 2), we performed *in vitro* gel shift analyses with nuclear extracts derived from CE BAT, CE liver, CE brain and TN BAT. All of the nuclear extracts exhibited protein-DNA interactions, suggesting that these protein-DNA binding motifs may be important regulatory sequences for *Ucp1* expression (Figures 12-19). Figure 12 represents a gel shift analysis of the enhancer region between -2,586 and -2,547 bp, containing the CRE3 binding motif. All of the nuclear extracts bound rather weakly to the oligonucleotide probe. While it is not surprising that protein-DNA interactions were observed in several tissues due to the involvement of c/AMP in many tissues for several different functions, the relative degree of binding in the TN BAT extract was not expected because thermoneutrality is known to repress *Ucp1* transcription. Figure 13 represents a gel shift assay of the enhancer region between -2,552 and -2,513 bp of the *Ucp1* gene. This region of the enhancer contained previously identified DNA binding motifs for PPAR γ and CRE2. Nuclear extracts from

all tissues tested produced diffuse gel shifts and the addition of CE brain extract produced two shifts. All of these protein-DNA interactions were partially competed by the addition of 200-fold excess unlabeled probe, suggesting that these protein-DNA interactions are specific to the probe. Figure 14 represents another gel shift assay in the enhancer region between -2,450 and -2,411 bp and contains a C/EBP binding site. All of the nuclear extracts again produced a diffuse band shift, despite repeated attempts to change the DNA binding conditions and the gel electrophoresis parameters. Liver nuclear extract produced the strongest shift and the weakest shift was observed using brain nuclear extract, consistent with the known pattern of C/EBP. A gel shift assay of the region between -2,416 and -2,377 bp is shown in Figure 15. The oligonucleotide probe used in this assay contains TRE and BRE2 DNA binding motifs observed previously and in our *in vivo* footprints. The resulting shifts are weak, but do suggest that this region binds nuclear proteins. Figure 17 represents a gel shift assay of region -184 to -145 bp of the promoter region of *Ucp1*. All of the extracts contain nuclear proteins which shifted the labeled oligonucleotide probe, but the addition of 200-fold excess unlabeled GSAP1 resulted in no removal of the shifts. This suggests that the binding of nuclear proteins in this region is non-specific. Figure 18 illustrates a gel shift of the promoter region between -164 to -125 bp. The oligonucleotide that we synthesized, GSAP2, contains the sequence located within an *in vivo* footprint that corresponds to the CRE4 DNA binding motif. All extracts produced a shift and the addition of 200-fold excess GSAP2 resulted in partial removal of the shifts. This suggests that the binding of

proteins to this region of the promoter is specific and that this region may be important to *Ucp1* expression.

4.3 Transgenic Analysis of the Promoter, Enhancer and Silencer Regions

We analyzed the transcriptional control of *Ucp1* through mutation analysis of several upstream untranslated regions. Mutated minigene constructs microinjected to generate transgenic mice and positive transgenic founders were crossed with Balb/cByJ +/+ mice to generate F₁ offspring. Expression of the transgenes was examined for tissue-specificity and cold inducible expression by northern analysis. The experiments were designed to answer three questions: (i) Will specific regulatory region deletions eliminate transcription of *Ucp1* in BAT of cold exposed mice? (ii) Do specific regulatory region deletions result in the *Ucp1* transgene constructs expression in mice adapted to thermoneutrality? (iii) Do any of these deletions result in expression of *Ucp1* in tissues other than BAT?

Transgenic evaluation of the NF-1 DNA binding site in the promoter region of *Ucp1* (*Ucp1*^{tg3.1} and *Ucp1*^{tg3.2}), resulted in no discernable differences in cold inducible or tissue-specific expression of the transgene compared to the endogenous gene (Figures 5, 6 and 7). These results were somewhat surprising since our laboratory previously observed cold-inducible *in vivo* footprints in this region (Chen, 1996). Our transgenic results therefore suggest that the 5' flanking DNA of *Ucp1*, between -166 to -163 bp, corresponding to the NF-1 hypersensitivity site of the *Ucp1* promoter is not required for cold inducible or tissue-specific expression of *Ucp1*.

Analysis of the minigene containing a deletion in the enhancer region (-2.5 to -2.3 kb, *Ucp1*^{tg4}), resulted in the complete loss of expression. This data supports the data of Kozak et al. (1994), suggesting that regulatory elements in the region located between -2,335 and -2,581 bp are critical for the expression of *Ucp1*.

Analysis of transgenic mice containing the minigene with the deletion of the proposed silencer region (between -991 and -230 bp, *Ucp1*^{tg6}), resulted in a tissue-specific and cold inducible expression no different from that observed for the endogenous *Ucp1* gene. The data from the *Ucp1*^{tg6} northern analysis is not consistent with results of Kozak et al.

(1994), who found that this region of DNA was associated with strong repression of *Ucp1* expression in cell culture. We initially hypothesized that this negative regulatory sequence could be responsible for the tissue-specific expression of *Ucp1*, and that through deletion of this element, we might see broader tissue distribution of *Ucp1*.

However, our results demonstrate that this sequence is not involved in BAT-specific expression or at least it does not act alone in limiting expression of *Ucp1* to BAT.

Our studies involving transgenic mice further refine the model of *Ucp1* expression. We know from previous work, that *Ucp1*^{tg1} (containing 3 kb of 5' flanking DNA) has the regulatory elements required for tissue-specific and cold inducible expression of the minigene. Moreover, *Ucp1*^{tg2} (containing 1.2 kb of 5' flanking DNA) results in no expression in BAT of cold exposed mice (Boyer and Kozak, 1991). This experiment suggested that there are important regulatory sequences between -3,000 and -1,200 bp.

We have now refined this regulatory region to elements located between -2,581 and -

2,335 bp, which have previously been predicted to be critical for tissue-specific and cold inducible expression of *Ucp1* (Figure 8 and 9). We have shown that this region has several potential regulatory regions where nuclear factor binding motifs can be found, namely CRE3, PPAR γ , CRE2, TRE, BRE2, and C/EBP (Chen, 1996). The region previously suspected of having silencer elements, between -991 and -230 bp, has no apparent effect on the expression of *Ucp1* in regards to tissue-specificity or cold adaptability (Figures 10 and 11). In addition, footprinting analysis implied a NF-1 hypersensitivity site located in the promoter region (Chen, 1996). Our work with *Ucp1*^{tg3.1} and *Ucp1*^{tg3.2} (Figure 5-7 and 19) show that -166 to -163 bp of the promoter region is not required for expression of *Ucp1* and that the protein that is binding to this section of the promoter is not conclusively NF-1.

5. References

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Table 1. List of oligonucleotides used in gel shift analysis**GSAE1**

-2586 CRE3 -2547
 TGAAGCTTGCTGTCACTCCTCTACAGCGTCACAGAGGGGTC

GSAE2

-2552 PPAR γ CRE2 -2513
 AGGGTCAGTCACCCTTGACCACACTGAACTAGTCGTCACC

GSAE3

-2450 C/EBP -2411

ACTGCTCTCTCCATTTATGAGGCAAACCTTTCTTTCACTTCC

mGSAE3 (mutated C/EBP site, but same flanking region as GSAE3)

ACTGCTCTCTCCATTACCTATTGCGCTTTCTTTCACTTCC

GSAE3mR (flanking region mutated right of C/EBP site)

ACTGCTCTCTCCATTATGAGGCAAATCCCTCCCTGTGCTC

GSAE3ML (flanking region mutated left of C/EBP site)

GTCATCTCTCTTTCCGTGAGGCAAACCTTTCTTTACCTCG

C/EBP (consensus C/EBP from Shuman et al., Science V. 249 p 774, 1990.)

TGCAGATTGCGCAATC

GSAE4

-2416 TRE BRE2 -2377

ACTTCCAGAGGCTCTGGGGGCAGCAAGGTCAACCCTTTCC

GSAP1

-184 NF-1 -145

CCAAATCAGAGGTGATGTGGCCAGGGCTTTGGGAGTGACG

mGSAP1 (mutated NF-1 site, but same flanking region as GSAP1)

CCAAATCAGAGGTGATGTTTACAGGGCTTTGGGAGTGACG

GSAP1mL (flanking region mutated left of NF-1 site)

TTGGGCTGAGAACAGCACGGCCAGGGCTTTGGGAGTTACC

GSAP1mR (flanking region mutated right of NF-1 site)

CCAAATCAGAGGTGATGTGGCTGAAATCCCAAAGACCCCC

NF-1 (consensus NF-1 from Mermod et al., 1989)

TGGCGCTATTTTGGATTGAAGCCAATATGACCGGCATAGT

GSAP2

-164 CRE4 -125

CAGGGCTTTGGGAGTGACGCGCGGCTGGGAGGCTTGCGC

- GSA = Gel Shift Analysis
- P = the Promoter region
- E = the Enhancer region

All of the probes were double stranded (only the top strand is shown) and 5' overhangs were end filled with Klenow. The underlined segments indicate the closest match to the consensus sequence for the nuclear factors that are located above the underlined sequences. All GSA probes are oligonucleotides synthesized for the purpose of *in vitro* analysis of footprinted regions.

Table 2. Abbreviation list

BAT	brown adipose tissue
BRE	brown fat regulatory elements
C/EBP	CCAAT enhancer binding protein
cAMP	cyclic AMP
CE	cold exposed
CRE	cAMP response elements
GSAE	gel shift assay, enhancer
GSAP	gel shift assay, promoter
NF-1	nuclear factor-1
PPAR γ	peroxisome proliferator-activated receptor γ
TN	thermoneutral
TRE	thyroid hormone response elements
<i>Ucp1</i>	Uncoupling protein 1 gene
UCP1	Uncoupling protein 1 protein
WAT	white adipose tissue

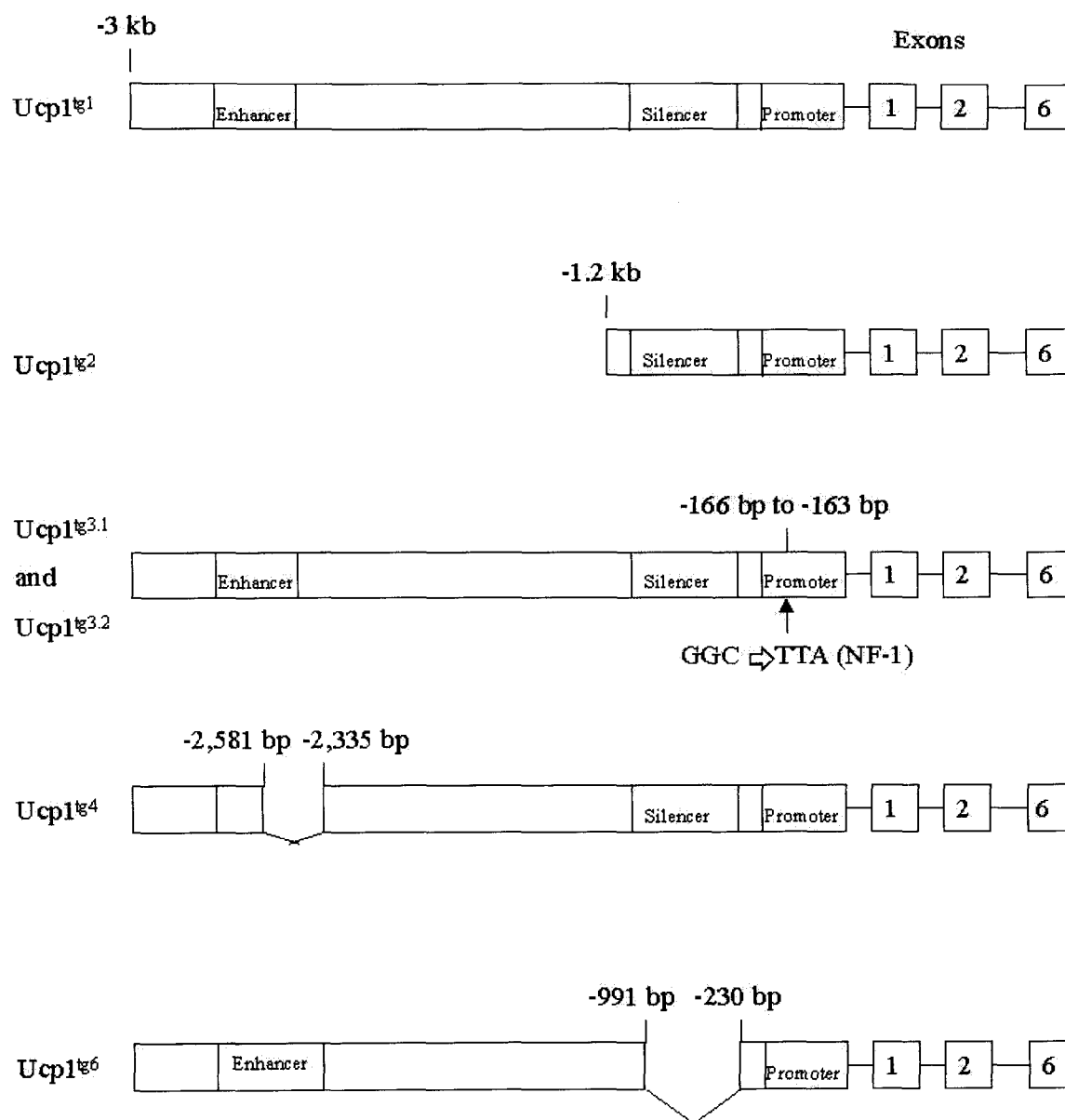


Figure 1. A map of the transgenic constructs. *Ucp1*^{tg1} is the endogenous 3 kb of 5' flanking DNA and therefore our control. Numbered boxes represent exons.

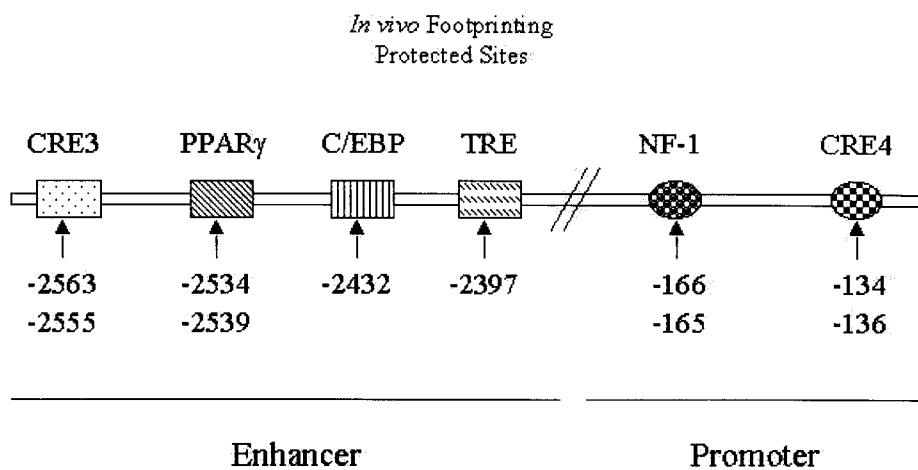


Figure 2. Map of *in vivo* footprinting performed by Chen, 1996.

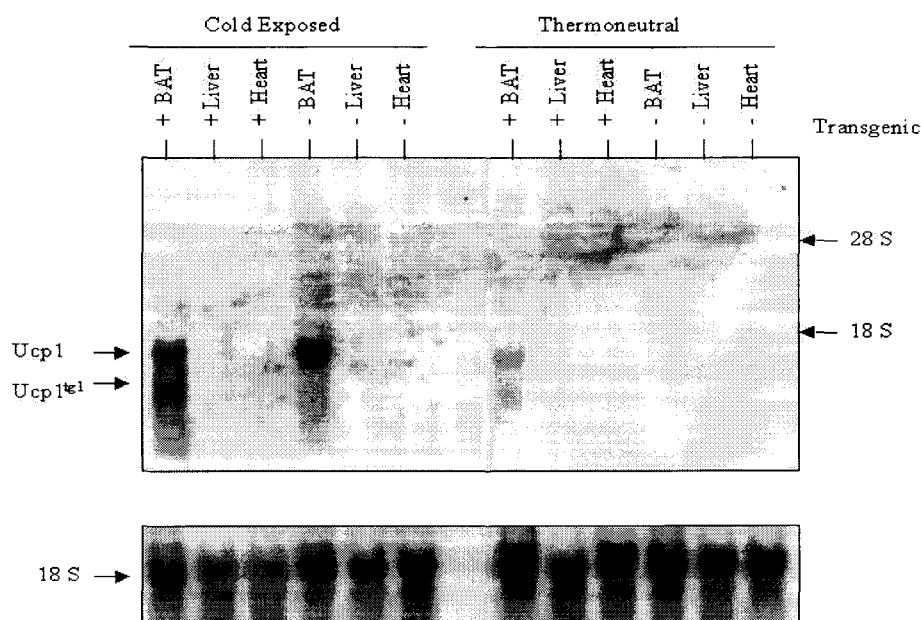


Figure 3. Cold exposed northern blot analysis of *Ucp1^{tg1}* mRNA. Twenty μ g total RNA were loaded per lane. Thermoneutral mice were acclimated to 29°C for three days. Cold exposed mice were thermoneutral acclimated to 29°C for three days and then exposed to 4°C for one hour. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below). Symbols: + indicates a transgenic mouse, - indicates a non-transgenic mouse.

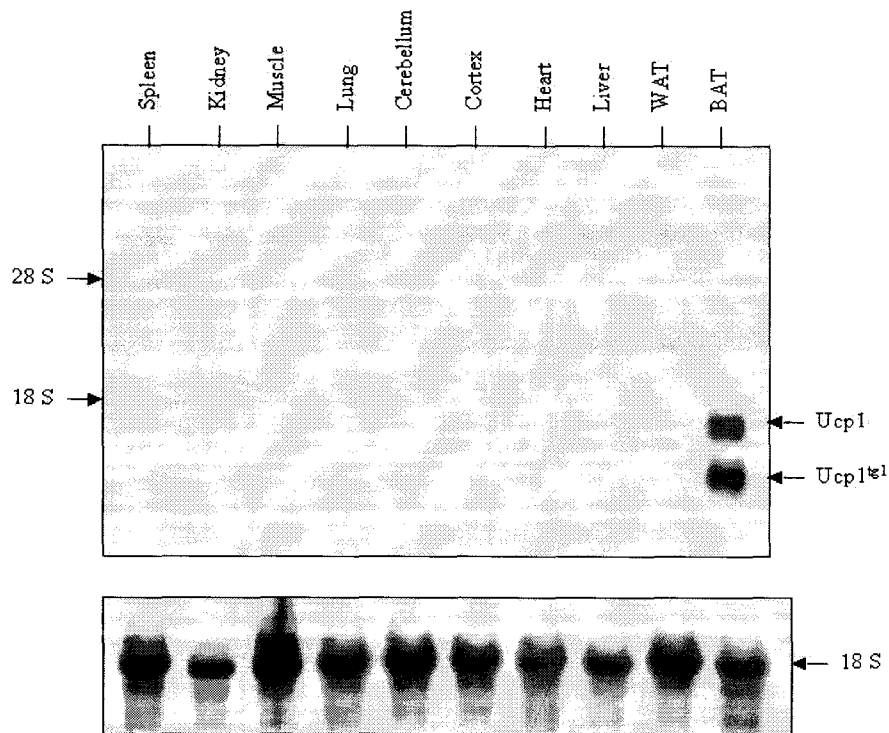


Figure 4. Tissue-specificity northern blot analysis of *Ucp1^{tg1}* mRNA. Twenty μ g total RNA were loaded per lane. Maximum transcription was induced by exposing mice to 4°C for 24 hours. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below).

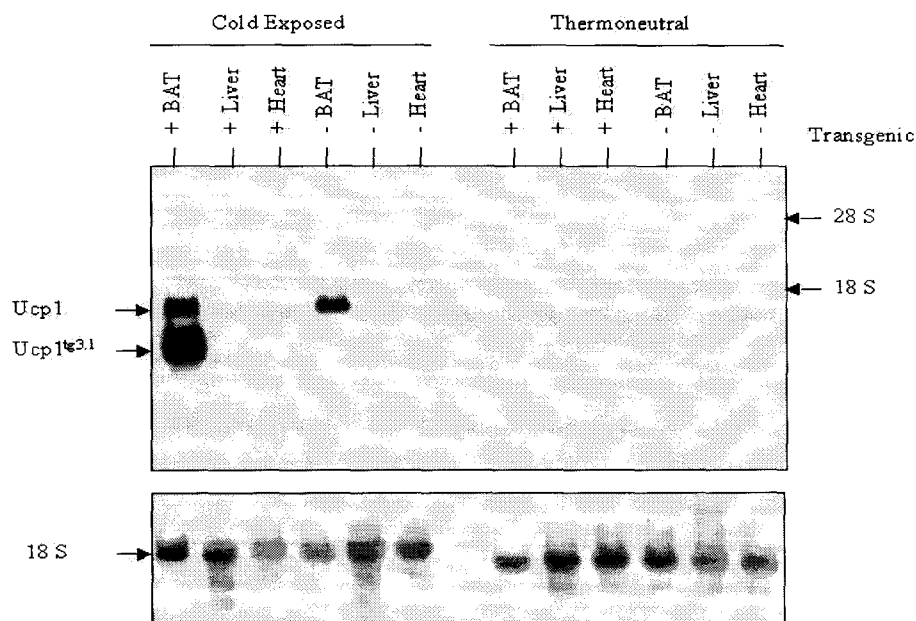


Figure 5. Cold exposed northern blot analysis of *Ucp1^{tg3.1}* mRNA. Twenty μ g total RNA were loaded per lane. Thermoneutral mice were acclimated to 29°C for three days. Cold exposed mice were thermoneutral acclimated to 29°C for three days and then exposed to 4°C for one hour. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below). Symbols: + indicates a transgenic mouse, - indicates a non-transgenic mouse.

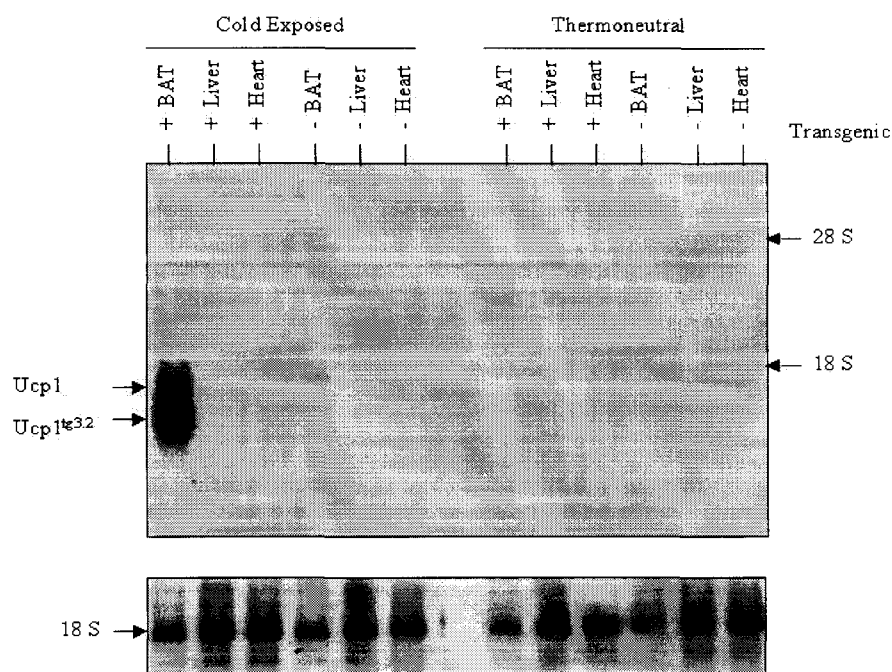


Figure 6. Cold exposed northern blot analysis of *Ucp1*^{tg3.2} mRNA. Twenty μ g total RNA were loaded per lane. Thermoneutral mice were acclimated to 29°C for three days. Cold exposed mice were thermoneutral acclimated to 29°C for three days and then exposed to 4°C for one hour. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below). Symbols: + indicates a transgenic mouse, - indicates a non-transgenic mouse.

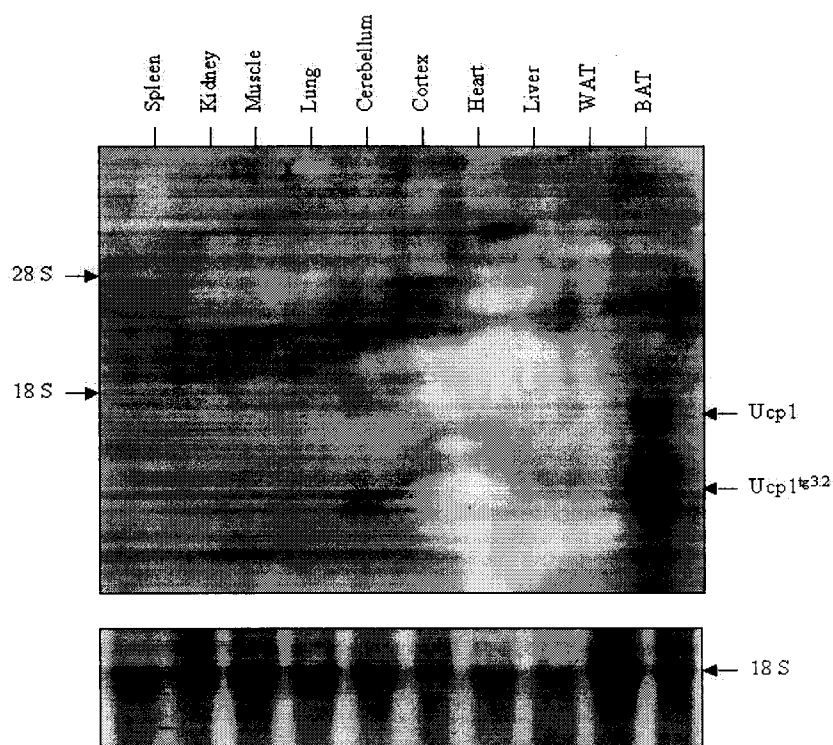


Figure 7. Tissue-specificity northern blot analysis of *Ucp1^{tg3.2}* mRNA. Twenty μ g total RNA were loaded per lane. Maximum transcription was induced by exposing mice to 4°C for 24 hours. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below).

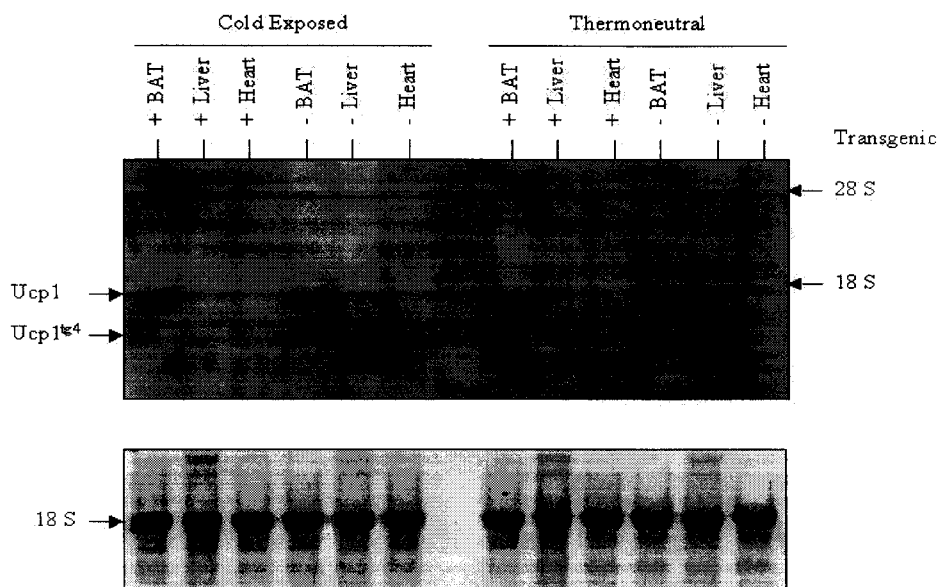


Figure 8. Cold exposed northern blot analysis of *Ucp1^{tg4}* mRNA. Twenty μ g total RNA were loaded per lane. Thermoneutral mice were acclimated to 29°C for three days. Cold exposed mice were thermoneutral acclimated to 29°C for three days and then exposed to 4°C for one hour. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below). Symbols: + indicates a transgenic mouse, - indicates a non-transgenic mouse.

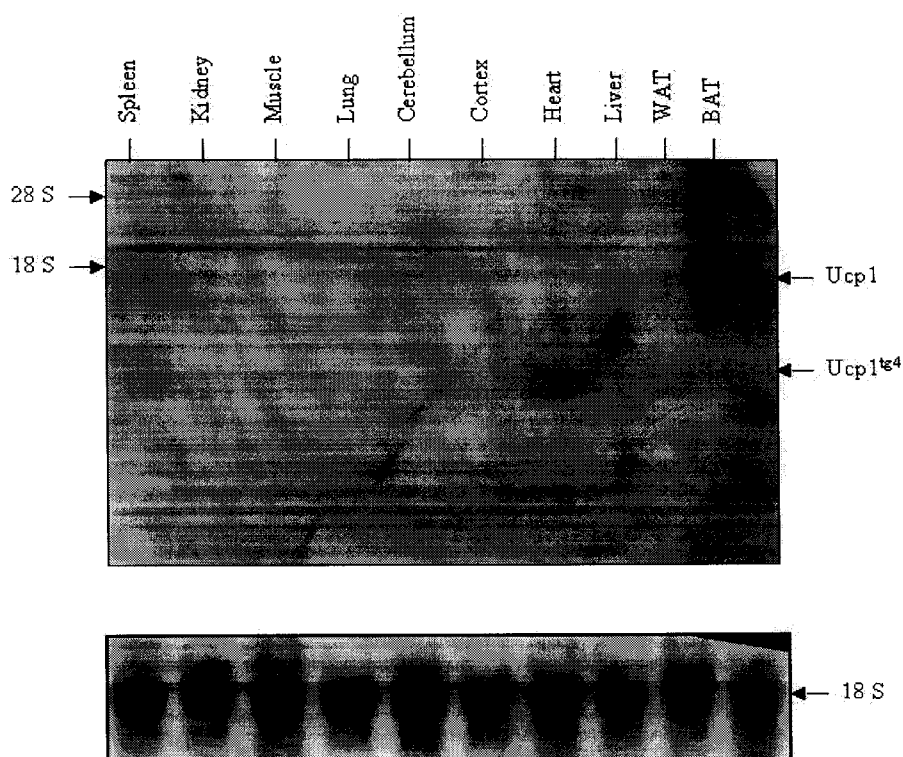


Figure 9. Tissue-specificity northern blot analysis of *Ucp1*^{tg4} mRNA. Twenty μ g total RNA were loaded per lane. Maximum transcription was induced by exposing mice to 4°C for 24 hours. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below).

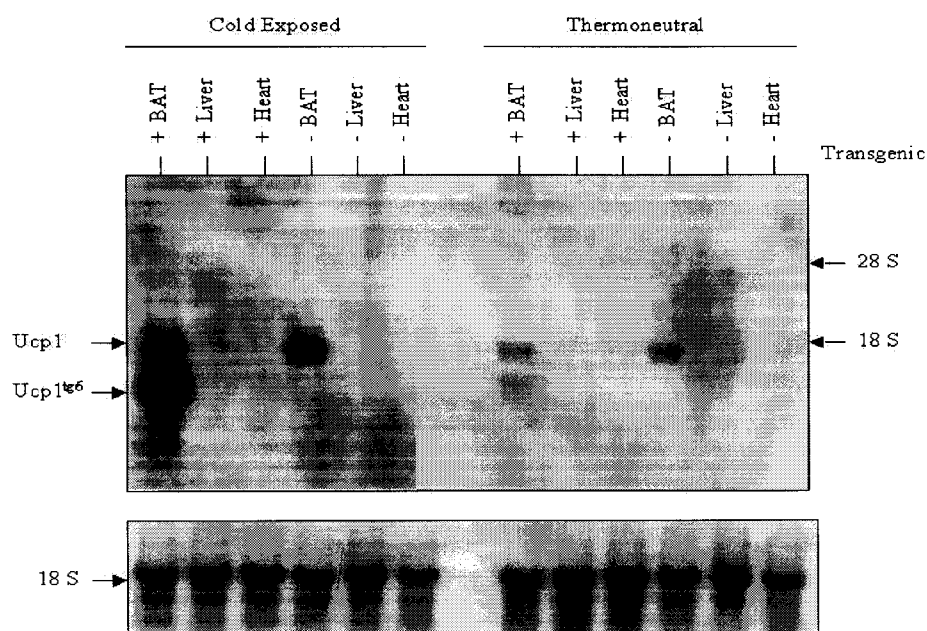


Figure 10. Cold exposed northern blot analysis of *Ucp1^{tg6}* mRNA. Twenty μ g total RNA were loaded per lane. Thermoneutral mice were acclimated to 29°C for three days. Cold exposed mice were thermoneutral acclimated to 29°C for three days and then exposed to 4°C for one hour. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below). Symbols: + indicates a transgenic mouse, - indicates a non-transgenic mouse.

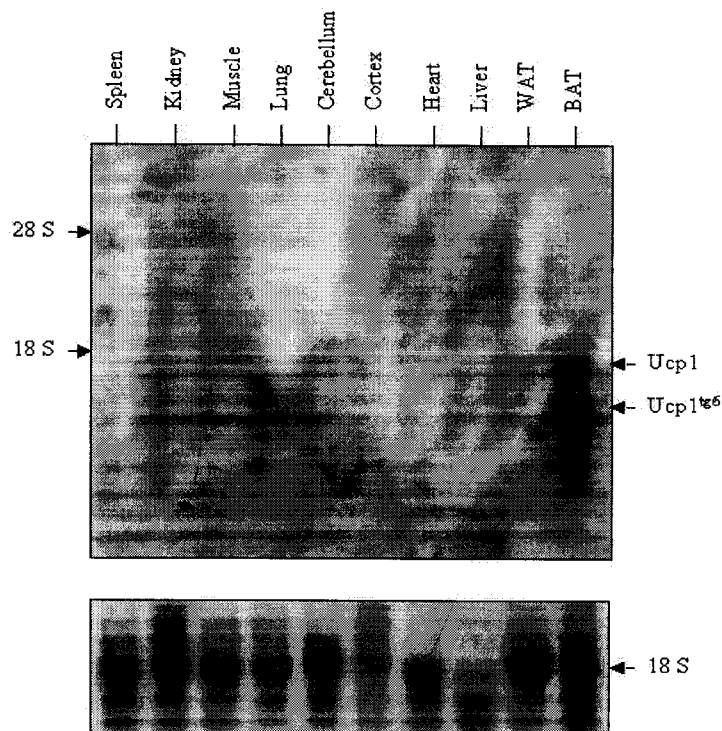


Figure 11. Tissue-specificity northern blot analysis of *Ucp1^{tg6}* mRNA. Twenty μ g total RNA was loaded per lane. Maximum transcription was induced by exposing mice to 4°C for 24 hours. Loading differences between lanes were resolved by normalization with a probe for the 18S RNA subunit (as shown below).

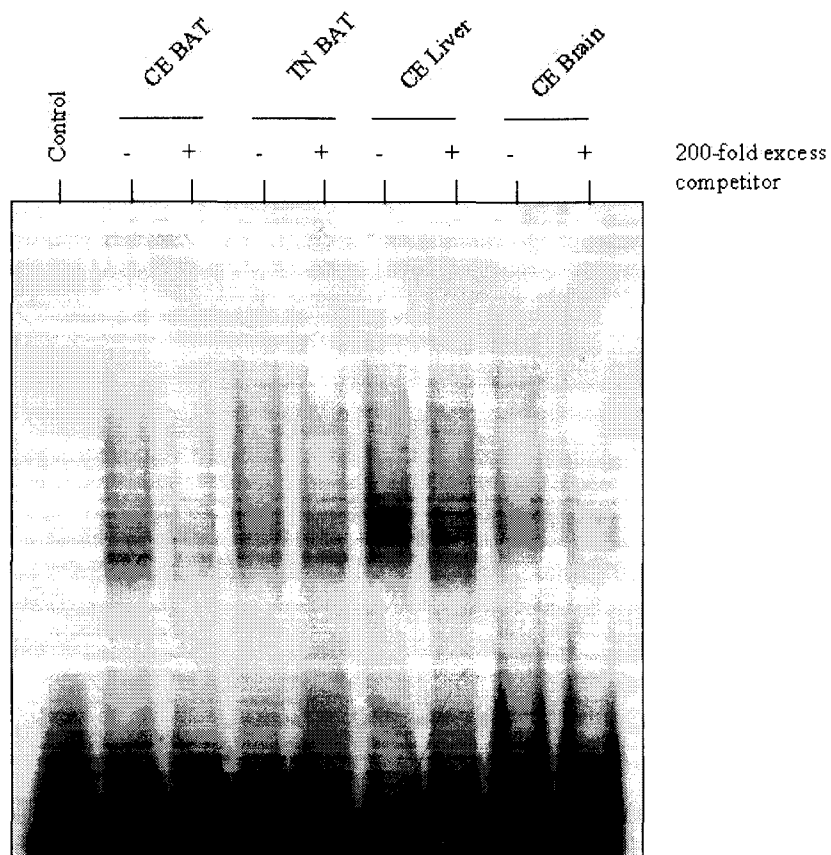


Figure 12. Gel shift assay of the enhancer region between -2,586 and -2,547 bp. Control lane is labeled probe without nuclear extract. Competitor was 200-fold excess unlabeled probe (GSAE1).

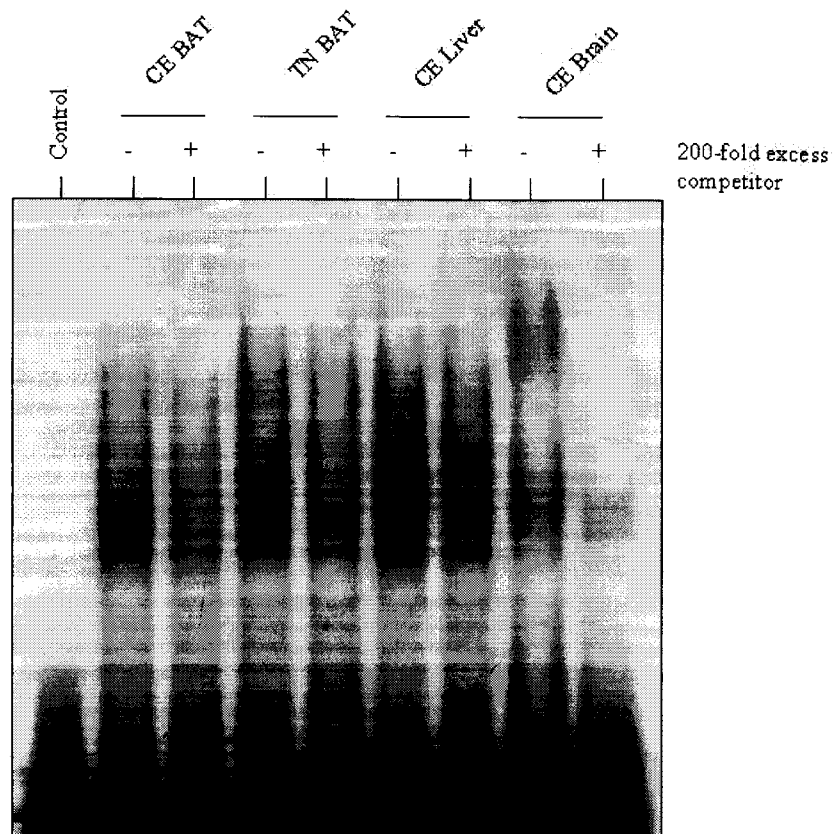


Figure 13. Gel shift assay of the enhancer region between -2,552 and -2,513 bp. Control lane is labeled probe without nuclear extract. Competitor was 200-fold excess unlabeled probe (GSAE2).

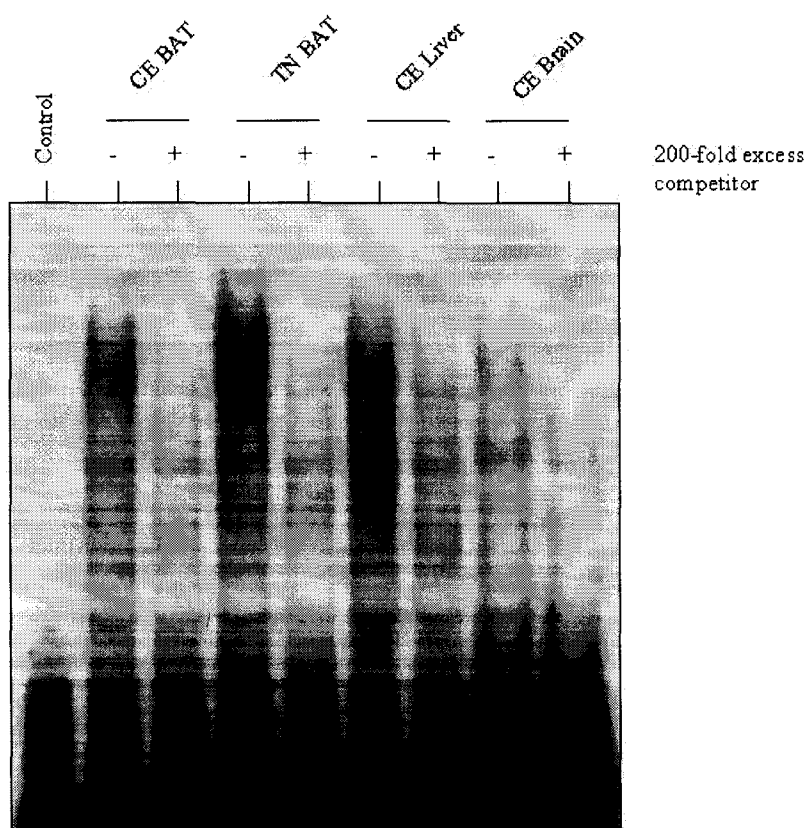


Figure 14. Gel shift assay of the enhancer region between $-2,450$ and $-2,411$ bp. Control lane is labeled probe without nuclear extract. Competitor was 200-fold excess unlabeled probe (GSAE3).

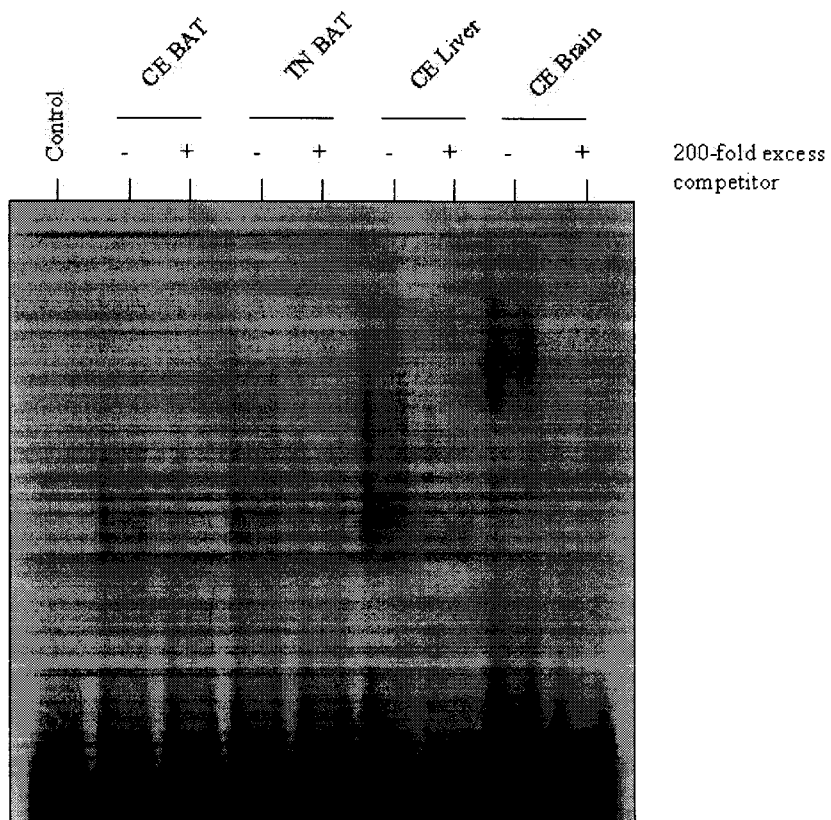


Figure 15. Gel shift assay of the enhancer region between -2,416 and -2,377 bp. Control lane is labeled probe without nuclear extract. Competitor was 200-fold excess unlabeled probe (GSAE4).

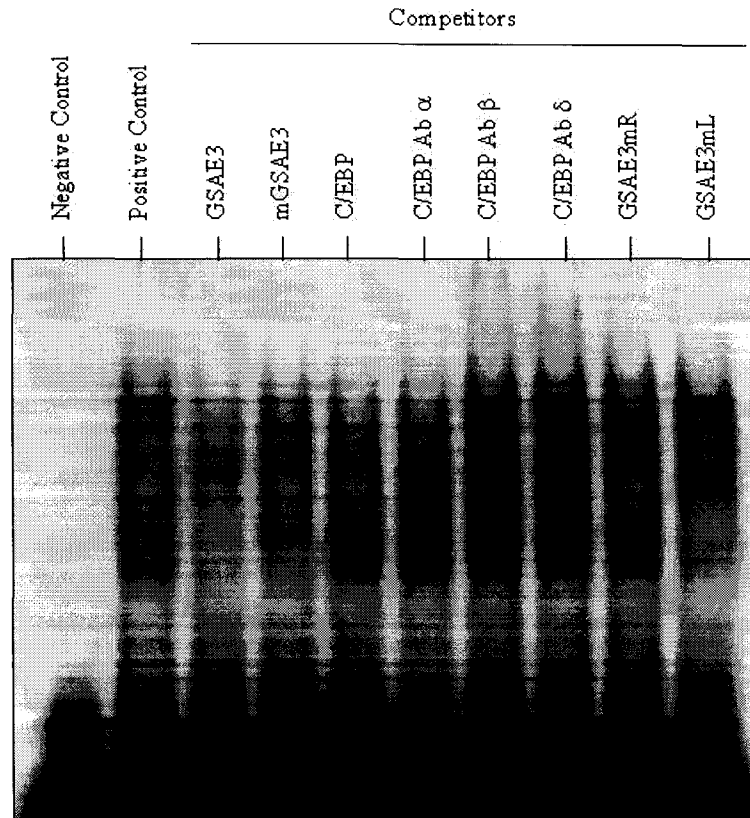


Figure 16. Gel shift competition assay of C/EBP. Probe used was GSAE3. Nuclear extract is CE BAT. Negative control lane is labeled probe without nuclear extract. Positive control is the labeled probe with nuclear extract. Competitors were unlabeled probes added in 200-fold molar excess. Lanes with antibodies do not contain competitors.

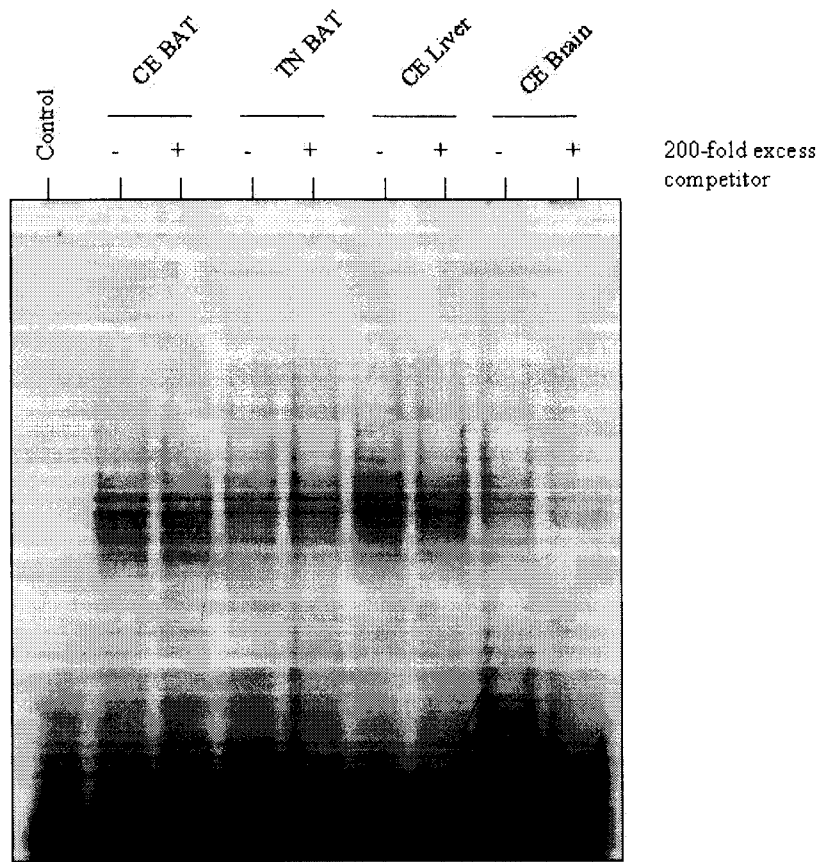


Figure 17. Gel shift assay of the promoter region between -184 and -145 bp. Control lane is labeled probe without nuclear extract. Competitor was 200-fold excess unlabeled probe (GSAP1).

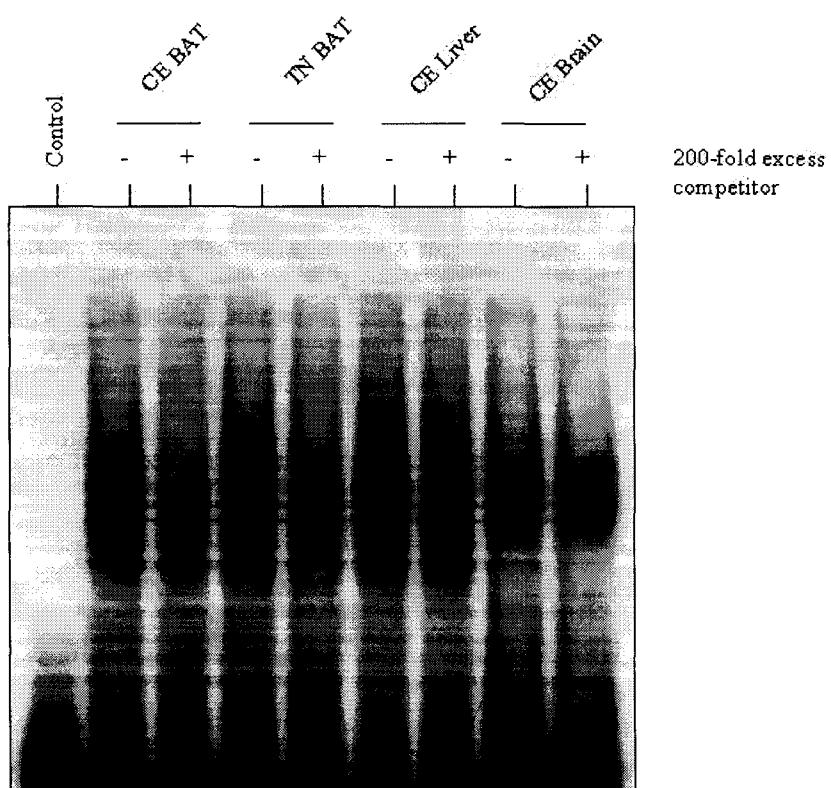


Figure 18. Gel shift assay of the promoter region between -164 and -125 bp. Control lane is labeled probe without nuclear extract. Competitor was 200-fold excess unlabeled probe (GSAP2).

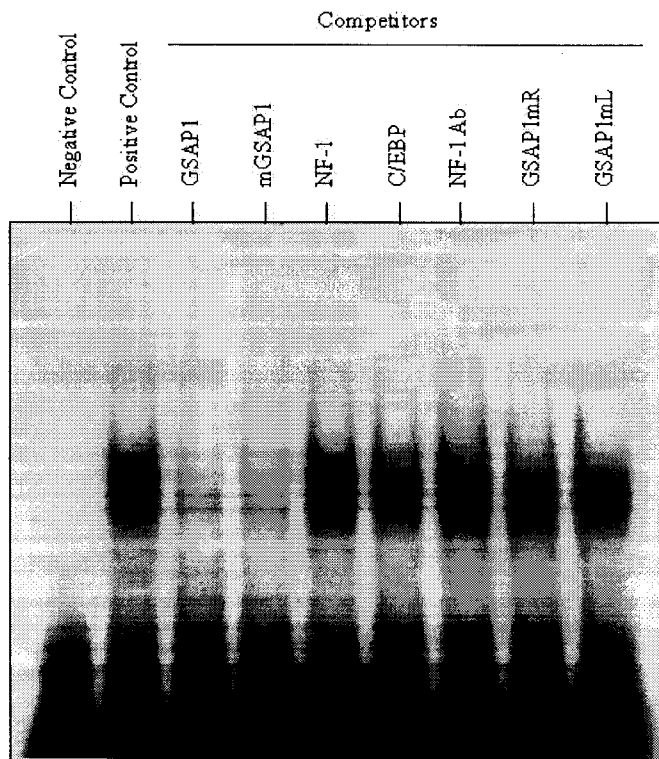


Figure 19. Gel shift competition assay of NF-1. Probe used was GSAP1. Nuclear extract is CE BAT. Negative control lane is labeled probe without nuclear extract. Positive control is the labeled probe with nuclear extract. Competitors were unlabeled probes added in 200-fold molar excess. Lanes with antibodies do not contain competitors.